Attorney's Docket No. 5051-484ip PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: P. Agris
Serial No.: 9/747,467
Filed: 12/22/2000

Group Art Unit: 1641
Examiner: CHEU, CHANGHWA J

For: METHODS AND COMPOSITIONS FOR DETERMINING THE PURITY OF CHEMICALLY SYNTHESIZED NUCLEIC ACIDS

Commissioner for Patents
Washington, DC 20231

RULE 132 DECLARATION OF PAUL F. AGRIS

I, Paul F. Agris, do hereby declare and say as follows:

1. I received my B.S. in Biology and Chemistry from Bucknell University in 1966 and my Ph.D. in Biochemistry from Massachusetts Institute of Technology in 1971. From 1971 to 1973 I was a Postdoctoral Fellow in the Yale University Department of Molecular Biophysics and Biochemistry. From 1973-1987 I was Assistant Professor, then Associate Professor, then Professor in the Division of Biological Sciences at University of Missouri-Columbia. Since 1988 I have been a Professor in the Department of Biochemistry at North Carolina State University. I am an inventor of the above-captioned patent application. My *Curriculum Vitae* is attached hereto as **EXHIBIT A.**

2. Mass spectrometry and capillary electrophoresis are used to detect aborted sequences of oligonucleotides and gel electrophoresis and chromatography (HPLC) to purify full-length oligomer. But these methods do not identify and quantify incompletely deprotected oligonucleotides or purify completely deprotected oligonucleotides from incompletely deprotected oligonucleotides. To address this problem, we developed in my laboratory, under my supervision, monoclonal antibodies (MAbs), ELISA, and dot-blot assays for the specific identification and quantification of the commonly used nucleic acid base- and sugar-protecting groups: benzoyl, isobutyryl, isopropylphenoxyacetyl, and dimethoxytrityl (Fu, C., Smith, S.,

Simkins, S.G. and Agris, P.F. (2002) Identification and quantification of protecting groups remaining in commercial oligonucleotide products using monoclonal antibodies. *Analytical Biochemistry* **306,** 135-143. (attached AS **EXHIBIT B**)). Using the MAb dot-blot assay, 5 of 16 commercial full-length DNA products obtained from eight different companies were found to have 1.0–5.2% of the benzoyl and isopropylphenoxyacetyl protecting groups remaining (**EXHIBIT B**).

3. Each MAb was capable of reproducibly and uniquely detecting 8–32 pmol of the respectively protected nucleoside in an intact DNA or RNA sample composed of 320–640 nmol of the deprotected nucleoside. In a direct comparison, HPLC nucleoside composition analysis of enzyme-hydrolyzed DNA was limited to the detection of 2–5 nmol of protected nucleoside. Each MAb characteristically recognizes the distinctive protecting group no matter the compound to which the protecting group is attached, DNA, RNA, cyclodextrin (Agris, P.F., Smith, S., Fu, C., and Simkins, S.G. (2002) QC in antisense oligo synthesis. *Nature Biotechnology* **20**, 871-872. (Attached as **EXHIBIT C**)). Thus, MAbs selectively identify and quantify picomoles of remaining protecting groups on antisense therapeutics and oligonucleotide diagnostics.

4. All of the statements made above of the undersigned declarants' own knowledge are true and all statements made on information and belief are believed to be true. The undersigned acknowledges that willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001), and may jeopardize the validity of the above-referenced application or any patent issuing thereof.

_____ _____

Paul F. Agris Date

Attachments: **EXHIBITS A-C**

Simkins, S.G. and Agris, P.F. (2002) Identification and quantification of protecting groups remaining in commercial oligonucleotide products using monoclonal antibodies. *Analytical Biochemistry* **306**, 135-143. (attached AS **EXHIBIT B**)). Using the MAb dot-blot assay, 5 of 10 commercial full-length DNA products obtained from eight different companies were found to have 1.0–5.2% of the benzoyl and isopropylphenoxyacetyl protecting groups remaining (**EXHIBIT B**).

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Paul F. Agris

3/08/04
Date

Attachments: **EXHIBITS A-C**

PAUL F. AGRIS
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EDUCATION:

B.S. (1966), BIOLOGY AND CHEMISTRY, BUCKNELL UNIVERSITY
PH.D. (1971), BIOCHEMISTRY, MASSACHUSETTS INSTITUTE OF TECHNOLOGY

RESEARCH AND/OR PROFESSIONAL EXPERIENCE:

1971	CONSULTANT TO NEW ENGLAND NUCLEAR CORPORATION
1971-73	POSTDOCTORAL FELLOW, YALE UNIVERSITY DEPARTMENT OF MOLECULAR BIOPHYSICS AND BIOCHEMISTRY
1973-87	ASSOCIATED SCIENTIST/CONSULTANT CANCER RESEARCH CENTER, COLUMBIA, MO
1973-77	ASSISTANT PROFESSOR, DIVISION OF BIOLOGICAL SCIENCES, U. OF MISSOURI-COLUMBIA
1977-83	ASSOCIATE PROFESSOR, DIVISION OF BIOLOGICAL SCIENCES, U. OF MISSOURI-COLUMBIA
1979-	PRESIDENT AND BOARD MEMBER, AMERICAN FOUNDATION FOR AGING RESEARCH
1980-90	BOARD MEMBER, AMERICAN FEDERATION FOR AGING RESEARCH
1980-81	VISITING SCIENTIST, BIOCHEMISTRY DEPT., OXFORD UNIVERSITY, ENGLAND
1981-82	VISITING SCIENTIST, OKLAHOMA MEDICAL RESEARCH FOUNDATION, OKLAHOMA CITY
1981-83	ASSOCIATE PROFESSOR, DEPART. OF MEDICINE, SCHOOL OF MEDICINE, U. OF MISSOURI
1983-87	PROFESSOR, DIVISION OF BIOLOGICAL SCIENCES, U. OF MISSOURI-COLUMBIA
1983-87	PROFESSOR, DEPARTMENT OF MEDICINE, U. OF MISSOURI- COLUMBIA
1985-87	DIRECTOR, DIV. OF BIOLOGICAL SCIENCES PROTEIN SEQUENCING LAB, U. OF MISSOURI
1988-93	HEAD, DEPARTMENT OF BIOCHEMISTRY, NORTH CAROLINA STATE UNIVERSITY, RALEIGH
1988-	PROFESSOR, DEPART. OF BIOCHEMISTRY, NORTH CAROLINA STATE UNIVERSITY, RALEIGH
1994-	CHAIRMAN, NCSU RNA BIOLOGY FACULTY
1996-	FOUNDER AND CHAIR RNA SOCIETY OF NORTH CAROLINA
1996-99	CONSULTANT TO SMITH KLINE, AND MERCK
2000	FOUNDER *NEOS DISCOVERY, INC.*
2000-2002	CSO *NEOS DISCOVERY, INC*
2002-	ADVISOR TO THE POLISH ACADEMY OF SCIENCES
2003-	CONSULTANT TO VERQ, INC.
2003-	RESEARCH AGREEMENT AND CONSULT WITH DHARMACON RNA TECHNOLOGIES

HONORS AND AWARDS:

N.S.F. UNDERGRADUATE RESEARCH FELLOW
N.I.H. PREDOCTORAL TRAINING GRANT FELLOW
POSTDOCTORAL FELLOW OF THE DAMON RUNYON MEMORIAL FUND FOR CANCER RESEARCH
POSTDOCTORAL FELLOW OF THE AMERICAN CANCER SOCIETY
NATIONAL JUNIOR CHAMBER OF COMMERCE OUTSTANDING YOUNG MAN OF 1978
FOGARTY SENIOR INTERNATIONAL FELLOW, 1980-81, UNIVERSITY OF OXFORD
SENIOR FELLOW, EXETER COLLEGE, OXFORD, 1980-
NATIONAL RESEARCH SERVICE AWARD SPECIAL FELLOW, 1981-82
U.S. NATIONAL ACADEMY OF SCIENCE EXCHANGE SCIENTIST WITH POLISH ACADEMY OF SCIENCES

EXHIBIT A

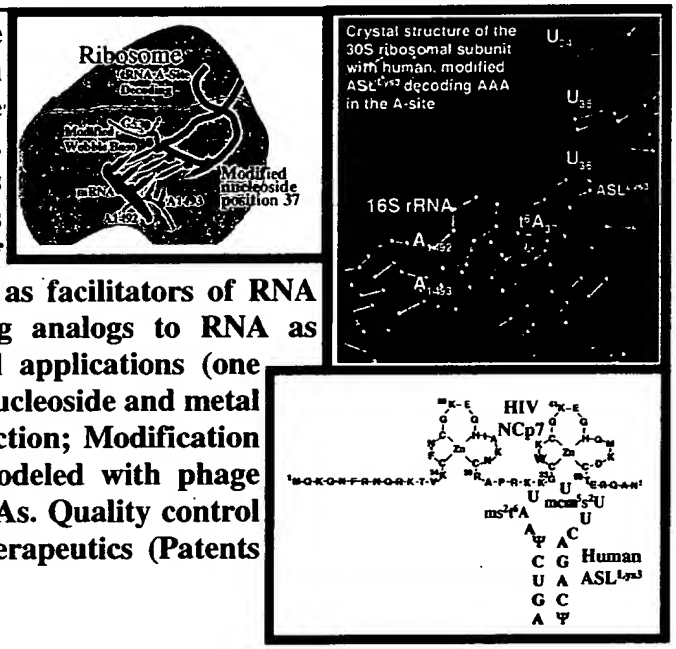
INSTRUCTIONAL ACTIVITIES:

Developed beginning undergraduate course for freshman and sophomore biochemistry and other science and non-science majors who lack college chemistry. Course based on molecular evolution allows students to learn chemistry, biochemistry and associated biology simultaneously.

Continuing to develop a multidisciplinary course in biophysical chemistry for beginning graduate students and for undergraduate seniors. The course brings instruction and instructors together from the disciplines of biochemistry, chemistry, chemical engineering, biomath/statistics, microbiology, and physics to teach macromolecular chemistry and structure with regard to function, functional folding of macromolecules, biometals, macromolecular dynamics and motors, functional genomics.

RESEARCH ACTIVITIES:

Contributions of nucleic acid chemistry, structure and dynamics to function in gene expression with application to biomedical sciences. Revision of the rules governing decoding of genomic information. RNA chemistry, structure-function relationships with particular emphasis on the modified nucleosides as protein recognition elements, and effectors of decoding, as tools and targets of intervention, and as facilitators of RNA folding. Modification in the design of functioning analogs to RNA as biological tools and targets with potential medical applications (one patent; two patent applications pending); modified nucleoside and metal ion contributions to nucleic acid structure and function; Modification dependent, RNA-protein functional interactions modeled with phage display selected peptide recognition of modified RNAs. Quality control in the synthesis of nucleic acid, oligonucleotide therapeutics (Patents pending; IP licensed).



PUBLICATIONS (SELECTED FROM ~130, SIX REVIEWS, SIX CHAPTERS, THREE BOOKS)

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Yarian,C, Marszalek, M, Sochacka, E, Malkiewicz, M, Guenther, R, Miskiewicz, A, Agris, PF (2000) $tRNA^{Lys}_{UUU}$ Species Require Anticodon Domain Modified Nucleosides for Watson-Crick and Wobble Codon Binding. ***Biochemistry* 39**:13390-13395.

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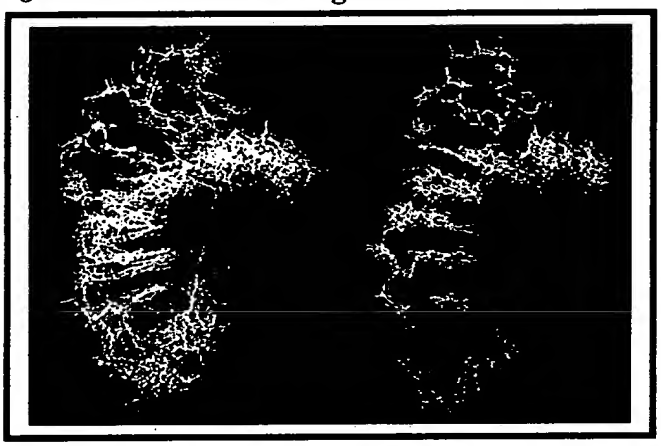
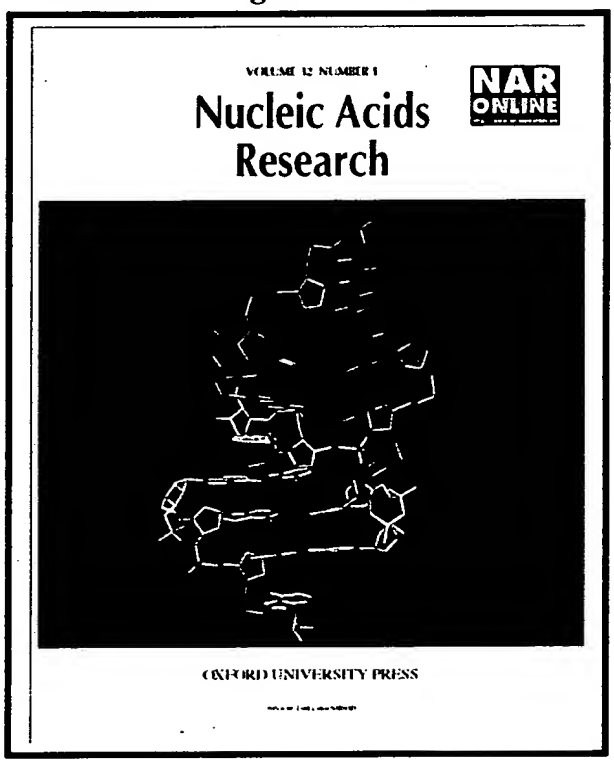
Agris, Paul F., Murphy, Frank, Malkiewicz, Andrzej, and Ramakrishnan, Venki (2003) Crystal Structure (~3 Å) of Ribosome Mediated, A-Site Decoding: tRNAs Hypermodified Purine-37 Is A Platform For The First Codon/Anticodon Base Pair. In preparation.

- **Journal Issue Cover Figure**



- **Generic Cover Figure for 2004**



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Identification and Quantification of Protecting Groups Remaining in Commercial Oligonucleotide Products Using Monoclonal Antibodies

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Quality control is paramount to reproducibly achieving oligonucleotide therapeutics and diagnostics of superior value. However, incomplete deprotection of nucleoside reactive groups after the automated chemical synthesis of oligonucleotides would result in diminished antisense activity and in erroneous array analysis of gene expression. Mass spectrometry and capillary electrophoresis are used to detect aborted sequences of oligonucleotides, but not to identify and quantify incompletely deprotected oligonucleotides. To address this problem, monoclonal antibodies (MAbs), ELISA, and dot-blot assays were developed for the specific identification and quantification of the commonly used nucleic acid base- and sugar-protecting groups: benzoyl, isobutyryl, isopropylphenoxyacetyl, and dimethoxytrityl. Each MAb was capable of reproducibly detecting 8–32 pmol of the respectively protected nucleoside in an intact DNA or RNA sample composed of 320–640 nmol of the deprotected nucleoside. In a direct comparison, HPLC nucleoside composition analysis of enzyme-hydrolyzed DNA was limited to the detection of 2–5 nmol of protected nucleoside. Using the MAb dot-blot assay, 5 of 16 commercial DNA products obtained from eight different companies were found to have 1.0–5.2% of the benzoyl and isopropylphenoxyacetyl protecting groups remaining. Thus, MAbs selectively identify and quantify picomoles of remaining protecting groups on antisense therapeutics and oligonucleotide diagnostics. © 2002 Elsevier Science (USA)

Key Words: **DNA; RNA; automated synthesis; protecting group detection; quality control.**

The automated chemical syntheses of DNA and RNA are quite simple in concept. Chemical synthesis of oligonucleotides requires that all but one of the chemically reactive functional groups of the nascent oligonucleotide chain remain protected during the ordered, sequential addition of nucleoside units (1–4). With completion of the synthesis process, the protecting groups such as the N^4-benzoyl-protection of cytidine are removed, typically with chemical agents. The base- and sugar-protected nucleoside 3′-phosphoramidite is the most widely used nucleotide chemistry for the automated chemical syntheses of DNA and RNA (Fig. 1) (1, 2). Many of the protecting groups applied to nucleoside phosphoramidite chemistry are found in other nucleic acid chemistries (3, 5) and in the synthesis of other polymers (6). Because automated oligonucleotide synthesis proceeds from the 3′ terminus to the 5′ terminus, only the 5′-OH-protecting group of the deoxyribose or ribose, usually a 4,4′-dimethoxytrityl (DMT)[2], is removed from the growing polynucleotide just prior to the next coupling reaction. In contrast, the exocyclic amines of the nucleobases are protected with various moieties, such as benzoyl (Bz), isopropylphenoxyacetyl (Ipr-Pac), and isobutyryl (Ibu) groups. These groups are resistant to DMT deprotection within the automated cyclic chemistries. Only at completion of polymer synthesis are the amine-protecting groups removed with an alkali treatment that also cleaves the

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[2] Abbreviations used: Bz, benzoyl; CE, capillary electrophoresis; CPG, controlled pore glass beads; DMT, dimethoxytrityl; ELISA, enzyme-linked immunosorbent assay; HAT, hypoxanthine, aminopterin, thymidine; Ibu, isobutyryl; Ipr-Pac, isopropylphenoxyacetyl; MALDI-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MAb, monoclonal antibody; TCA, trichloroacetic acid; *t*-BDMSi, *t*-butyldimethylsilyl; INT mm², integrated density of tested sample area minus that of an equal area of blank dots.

0003-2697/02 $35.00

EXHIBIT B

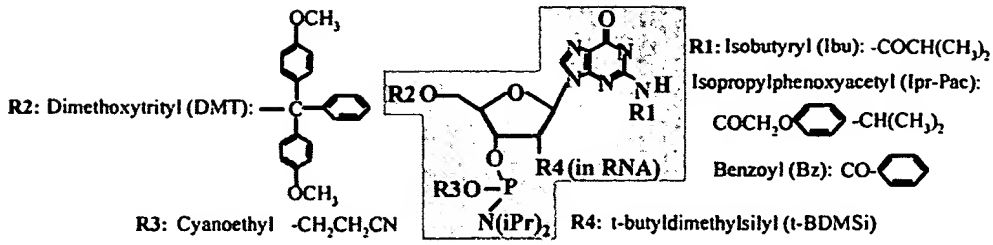

FIG. 1. Adenosine phosphoramidite and the commonly used protecting groups in DNA and RNA chemical syntheses. R1 represents the exocyclic amine-protecting groups: Bz, Ibu, and Ipr-Pac. R2 is the 5'-OH-protecting group: DMT. R3 represents the phosphate-protecting group: cyanoethyl. R4 is 2'-OH-protecting group: *t*-BDMSi. The $N(iPr)_2$ group is the (*N,N*-diisopropyl) moiety of the phosphoramidite. The chemical description for an *N*2-benzoyl-protected deoxyadenosine phosphoramidite is 5'-dimethoxytrityl-*N*-benzoyl-2'-deoxyadenosine,3'[(2-cyanoethyl)-(*N,N*-diisopropyl)].

product DNA or RNA from its 3' linkage to a solid support, such as controlled pore glass beads (CPG). In RNA synthesis, each ribose 2'-OH is protected to negate formation of branched-chain polymers, and this protecting group (Fig. 1) is removed after completion of the synthesis.

Though exceedingly efficient, neither the coupling reaction producing the growing polymer chain nor the subsequent deprotection of the full-length oligonucleotide is complete. Various factors including reaction conditions and reagent lifetime affect the coupling reaction and the chemical removal of protecting groups (7–9). The product consists mostly of deprotected, full-length DNA or RNA but also contains incompletely deprotected, full-length and aborted sequences. Most molecular biology experiments *in vitro* are accomplished with sufficient excess of oligonucleotide that results are not affected by incompletely deprotected oligomers constituting as much as 10% of the sample (7–9). In contrast, incomplete deprotection below the detection limit of present methods could produce spurious and inexplainable results *in vitro* and *in vivo* and lead to erroneous conclusions about the efficacy of nucleic acid antisense therapeutics (10–12). Incompletely deprotected diagnostic DNA arrays (chips) also could lead to mistaken conclusions about genetic variability and/or gene expression. Therefore, the U.S. Food and Drug Administration has expressed regulatory concerns and the Center for Drug Evaluation and Research established a Complex Drug Substances Coordinating Committee to investigate and suggest good laboratory practices and reporting requirements for oligonucleotide therapeutics.

Unfortunately, no simple, reproducible, sensitive, inexpensive, and thus, broadly available analytic method existed to identify each and every protecting group that may remain either in a sample in solution or on a diagnostic chip. HPLC nucleoside composition analysis is able to identify and quantify remaining protecting groups, but is insensitive because it depends on UV diode array detection for identification and quantification (13). Capillary electrophoresis (CE) (14), mass spectrometry (MS) (15, 16) and MALDI-MS (16) can detect the aborted sequences, but are not easily adapted to identifying and quantifying remaining protecting groups. HPLC-MS of the oligonucleotide's constituent nucleosides (17) is sensitive, but is not widely available.

We have generated several monoclonal antibodies (MAb) that are specific to the commonly used protecting groups of the exocyclic amines of nucleosides, Bz, Ipr-Pac, and Ibu, and monoclonal antibodies that recognize the 5'-OH protecting group, DMT, of full-length oligomers. Using these MAbs, a dot-blot assay and a microplate ELISA have been developed for identification and quantification of protecting groups that remain in standard, intact DNA and RNA oligonucleotide samples and commercial oligonucleotide products.

MATERIALS AND METHODS

Preparation of Oligonucleotides

Oligonucleotides used for generation and characterization of the monoclonal antibodies in this study were synthesized at the Nucleic Acid Facility of North Carolina State University employing phosphoramidite chemistry and CPG supports from Glen Research (Sterling, VA). An ABI DNA/RNA synthesizer (Model 394; Applied Biosystems, Foster City, CA) was used for the syntheses according to the manufacturer's protocol. After synthesis, the DMT group at the 5'-OH of the final nucleotide was removed from the oligomer while on the synthesizer, using TCA, except for those oligomers used in characterizing the anti-DMT antibody. CPG supports bearing the synthesized oligomer chains were treated at room temperature overnight to cleave the oligomers from the beads, yet retain the base-protecting groups. To remove the base-protecting groups, as well as the β-cyanoethyl group, oligonucleotides were treated with 30% NH_4OH (Fisher Scientific, Pittsburgh, PA) at 65°C for 6 h. RNA was treated with tetrabutylammonium fluoride (Sigma, St. Louis, MO) to remove the *t*-BDMSi groups from the 2'-OHs. Thereafter, the released oligomers were desalted,

dried, redissolved in water, aliquoted, and stored in −20°C. In this paper, the standards containing protecting groups were homopolymeric 20-mers designated oligo Bz-dC, Bz-dA, Ipr-Pac-dG, and Ibu-dG to denote protected nucleoside. Stringent NH_3 treatment removes the protecting groups leading to deprotected, negative control oligomers designated oligo dC^{Bz}, dA^{Bz}, $dG^{Ipr-Pac}$, and dG^{Ibu}, with the protecting group of the phosphoramidite precursors in superscript. A 20-mer synthesized with protected dT phosphoramide acted as a control in that no base-protecting group is used in its synthesis. The DMT was removed with TCA and the β-cyanoethyl group removed with NH_3, and the oligomer was designated oligo dT. Oligo dT retaining the DMT group was designated oligo DMT-dT or oligo Trebler-dT, where Trebler (18) is a moiety of three DMTs with a linker. RNA samples containing the Bz, Ipr-Pac, and Ibu groups were synthesized (2) and treated appropriately to obtain both the protected and the deprotected oligomers as the standard and negative control samples, respectively.

To evaluate the ability of the MAbs and assays to identify and quantify protecting groups remaining on commercial oligonucleotide products, two oligomers were purchased from each eight different companies: Alpha DNA, Montreal, Quebec, Canada; Integrated DNA Technologies, Coralville, Iowa; Life Technologies, Rockville, Maryland; Operon Technologies, Alameda, California; Research Genetics, Huntsville, Alabama; Sigma Genosys, The Woodland, Texas; The Great American Gene Company, Romona, California; and The Midland Certified Reagent Company, Midland, Texas. One of the DNA sequences was composed of 10 repeats of dA–dC, while the other contained 10 repeats of dG–dT. The DNAs were synthesized on the smallest column size advertised by the company at that time (40-, 50-, or 200-nmol scales), desalted, and shipped by express mail.

Nucleoside Composition Analysis

Mononucleosides and protected mononucleosides were purchased as standards for HPLC nucleoside composition analysis of the oligomers and included 2′-dA, 2′-dT, 2′-dC, and 2′-dG (Sigma Chemical Co.), and Bz-2′-dA (Aldrich Chemical Co., Milwaukee, WI). N^2-Ibu-dG and N^2-Ipr-Pac-dG were gifts from Monomer Sciences (New Market, MA). Six different concentrations of each protected nucleoside in triplicate were used to obtain the individual extinction coefficients under the conditions of the HPLC nucleoside composition analysis. Then, the lowest detection limit by the UV-monitored HPLC method was determined. HPLC nucleoside composition analysis with UV diode array detection "on the fly" identified and quantified protecting groups on the positive references and confirmed the lack of protecting groups on negative controls as below the detection limit of the HPLC method (13). Peak retention times and UV spectra were used for identification of nucleosides, whereas peak areas and nucleoside extinction coefficients established the amount of protected nucleoside present (13). For the standard oligo Bz-dC, Bz-dA, Ipr-Pac-dG, and Ibu-dG samples, the percentage of protected nucleoside, the nanomoles of protecting group per A_{260} unit of polymer, and the lowest level of detection of each by HPLC are listed in Table 2.

The extinction coefficients of DMT and Trebler were determined with six different concentrations of the monomer DMT-dT and of Trebler-dT in triplicate treated with acid and the absorbance was read at 495 nm. The 5′-OH protecting group, DMT, was detected by treatment of samples in solution and on a nitrocellulose membrane support with TCA. The amount of DMT or Trebler could be quantified precisely in solution by establishing a standard curve of absorbance (495 nm) with different concentrations of DNA/RNA synthesis grade DMT-chloride (ChemGenes Co., Ashland, MA). Specific amounts of DMT, or Trebler, were applied to a series of dots on a nitrocellulose membrane and treated with TCA, and the dot densities were quantified with a Personal Densitometer SI (Molecular Dynamics–Amersham Biosciences, Piscataway, NJ). The amount of DMT group per A_{260} unit of oligo DMT-dT and Trebler-dT and the lowest detection limit of DMT by visual spectroscopy are listed in Table 2.

Generation of Monoclonal Antibodies

Standard hybridoma production and culture techniques were employed at the Hybridoma Facility of North Carolina State University. Murine-derived MAbs against Bz (Bz-MAb), Ibu (Ibu-MAb), Ipr-Pac (Ipr-Pac-MAb), and DMT (DMT-MAb) were obtained through polyethylene glycol-mediated fusion of spleen cells from hyperimmunized female, 12-week-old BALB/c mice (Charles River, Wilmington, MA) with myeloma P3-X63-Ag8.653 cells (American Type Culture Collection, Manassas, VA). Each mouse received 10 intraperitoneal (ip) immunizations of 50 μg antigen performed at 2-week intervals. The mice were rested for 2 months and a final 50-μg boost was injected ip 5 days prior to fusion. Polyclonal antibody-containing plasma was collected on days 0, 34, and 81 via retro-orbital bleeds for Bz-, Ibu-, and Ipr-Pac-specific antibody titer analysis. The mice were anesthetized prior to bleeding with intramuscular injection of 2 mg of Ketaset (Fort Dodge Animal Health, Fort Dodge, IA) and 0.5 mg of Rompun (Miles, Shawnee Mission, KS). The production of polyclonal antibody and the generation of hybridomas secreting MAb against Bz, Ibu, and Ipr-Pac protecting groups were repeated with a second

set of mice. Other mice were hyperimmunized with a DMT derivative. Seven immunizations were given over a 3-month period. A final 100-μg boost was injected 5 days prior to fusion. Polyclonal antibody containing plasma was collected on days 0, 38, and 81 for DMT-specific antibody titer analysis. Each hybridoma secreting MAb specific to Bz, Ipr-Pac, Ibu, or DMT was selected from approximately 1000 cultures and cloned three times. Standard HAT (Sigma Chemical Co.) selection was used to generate hybridoma growth. ELISA and dot-blot screening assays, as described below, were used to detect antigen-specific antibody during fusion and cloning procedures. Each hybridoma used in this study was limit dilution cloned three times at one cell/well. During cloning procedures, wells with antigen-specific antibody and a single isolated colony were selected for further development. Static exhausted supernatant was generated from each hybridoma and used for the various procedures described herein.

After subcloning, the lines were stable and have remained so for 15 months. During that period, these established hybridomas were used for production of MAb-containing culture supernatants three to five times, including lots as large as 500 ml of reagent. Cell culture supernatants were collected and stored at −20°C. Antibody isotypes were determined using a mouse-hybridoma subtyping kit (Boehringer Mannheim Corp., Indianapolis, IN), according to the manufacturer's protocol. The antibodies specific to amine-protecting groups were IgM with κ light chains, whereas the antibody specific to DMT presented a double isotype of IgM and IgG1 with κ light chains. Cell culture supernatants were used directly for assays.

Dot-Blot Assay

Oligonucleotide samples were blotted to nitrocellulose membranes (0.2 μm; Bio-Rad, Hercules, CA) through a manifold (Minifold; Schleicher & Scheull, Inc., Keene, NH) connected to an aspirator. The membranes were subjected twice to UV cross-linking at 254 nm, 1200 μJ/cm^2 (UV Crosslinker, FB-UVXL-1000; Fisher Scientific, Pittsburgh, PA). Thereafter, the blocking of nonspecific binding, washing, incubation with hybridoma cell culture supernatants, and alkaline-phosphatase-conjugated goat anti-mouse IgM or IgG reagent, as well as the subsequent enzyme–substrate reaction, were conducted according to the reagent manufacturer's protocol (Sigma Chemical Co.). TBS buffer (Tris 25 mM, pH 7.2, 150 mM NaCl) was used for blotting samples on membranes, TBS plus 0.1% Tween 20 (TBST) for washing, and 1% casein (Sigma Chemical Co.) in TBST for blocking and dilution of the enzyme-conjugated secondary antibodies. Blot-dot densities were determined with an imaging device (Quantity One, Bio-Rad). In the range of 0.2 to 0.8 (INT mm^2, the integrated density of the tested sample area minus that of an equal area from blank dots), there was a linear relationship between dot density and nmol of protecting group in the standard oligomers from which standard curves of response were generated. To demonstrate that the oligomers were bound to the nitrocellulose membrane, oligonucleotides were synthesized with 3′-terminal biotin (Glen Research, Sterling, MA). After the biotinylated oligomers were cross-linked to nitrocellulose, their presence was detected with an avidin–phosphatase conjugate (Sigma Chemical Co.). The extent of any nonspecific binding to the membrane was determined with a series of controls that resulted in dots no different in color than blank dots. Controls included oligomers plus phosphatase-conjugated anti-mouse antibody and substrates, oligomers plus substrates, MAbs plus phosphatase-conjugated anti-mouse antibody and substrates, MAbs plus substrate, conjugated anti-mouse antibody plus substrates, and oligomers plus myeloma cell culture, phosphatase-conjugated anti-mouse antibody, and substrates.

ELISA

Oligomers with 3′-terminal biotin were bound to strepavidin-coated microtiter plates (Boehringer Mannheim Corp.) by incubation at room temperature for 2 h. Plates were blocked, washed, and incubated with hybridoma cell culture supernatants and peroxidase-conjugated anti-mouse whole immunoglubin (Sigma Chemical Co.), and enzyme–substrate reaction was conducted according to the manufacturer's protocol (Sigma). The results were assessed with a computer-controlled reader (Bio-Tek Instruments, Winooski, VT).

Mass Spectrometry

Electrospray and MALDI-MS of protected and deprotected DNA samples were conducted according to published protocols (19, 20).

RESULTS

Specificity of Monoclonal Antibodies

In order to use antibodies for the purpose of detecting and identifying protecting groups remaining on oligonucleotides, the antibodies must be group specific. Standard and negative control oligonucleotides, respectively, with and without protecting groups (Fig. 1), were synthesized and used to assess the specificity of each monoclonal antibody. In a MAb dot-blot assay, the Bz-MAb reacted with the oligo Bz-dC and Bz-dA standards. The Bz-MAb did not react with the deprotected, negative controls, oligo dC^{Bz} and oligo dA^{Bz}. Moreover, the antibody did not react with any of the other standard or negative control oligomers, such as oligo Ipr-

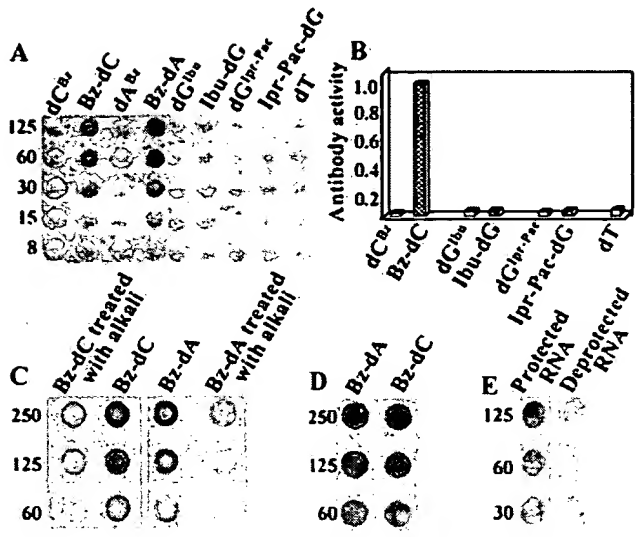

FIG. 2. Specificity of the Bz-MAb in a dot-blot assay and ELISA. Specificity of the Bz-MAb was determined by assessing its ability to distinguish the various protecting groups on homopolymeric DNA standards oligos Bz-dC, Bz-dA, Ibu-dG, and Ipr-Pac-dG and negative controls oligos dC^{Bz}, dA^{Bz}, dG^{Ibu}, $dG^{Ipr-Pac}$, and dT. (A) Dot-blot assay exhibiting the specificity of the Bz-MAb. Oligonucleotides were applied to the nitrocellulose membrane in the range of 8–125 pmol per dot. (B) ELISA demonstrating specificity of the Bz-MAb. Biotinylated (3') oligonucleotide standards were coupled to an avidin microplate and reacted with the Bz-MAb according to the procedure described under Materials and Methods. Antibody activity is normalized to reactivity, with Bz-dC being 1.0. (C and D) Dot-blot assays demonstrating that alkali-treated, standard oligos Bz-dC and Bz-dA are no longer recognized by the antibody and that the Bz-MAb recognizes oligo Bz-dC and oligo Bz-dA equally well. In (D), the amounts of Bz-protecting group per oligo Bz-dC and Bz-dA were determined with an HPLC nucleoside composition analysis, normalized with negative control oligonucleotide, and applied to the membrane in the range of 60–250 pmol DNA per dot. Equivalent numbers of Bz groups, as determined by HPLC, resulted in equivalent dot densities. (E) A dot-blot assay demonstrating that the Bz-MAb recognizes Bz on RNA.

Pac-dG, Ibu-dG, DMT-dT, or oligo $dG^{Ipr-Pac}$, dG^{Ibu} and dT (Fig. 2A). In an ELISA, the identical specificity was observed (Fig. 2B). After the oligo Bz-dC and oligo Bz-dA standards were treated with alkali to remove the Bz group, the antibody no longer recognized the oligomers (Fig. 2C). HPLC nucleoside composition analysis of the alkali-treated DNA samples also was incapable of detecting any remaining Bz-dC or Bz-dA mononucleosides (data not shown). The Bz-MAb detected the Bz-protecting group equally well whether on oligo Bz-dC or on oligo Bz-dA (Fig. 2D). Thus, the nature of the nucleobase had no significant effect on interaction of the antibody and its target, the Bz group. Furthermore, the Bz-MAb recognized Bz groups on the RNA standard, but not on the respective negative control RNA oligomer (Fig. 2E). Thus, antibody specificity for the Bz group was not affected by the nature of the nucleic acid backbone.

The specificities of the Ipr-Pac-MAb and Ibu-MAb were characterized similarly with the dot blot assays (Table 1) and ELISAs (not shown). However, the Ipr-Pac-MAb demonstrated a dual specificity for both Ipr-Pac and Ibu groups (Table 1). We postulate that the Ipr-Pac-MAb epitope is the isopropyl moiety present in both protecting groups (Fig. 1). Specificity of the DMT-MAb for the 5'-terminal DMT group and its triple derivative, Trebler, was similarly assessed (Table 1). The nature of the nucleobase of the deoxyribose to which DMT was coupled was immaterial to the recognition. Because the DMT-group linkage to the 5'-OH of the nucleic acid is labile, its presence during the dot-blot assay was monitored. Presence of the DMT moiety on the nitrocellulose membrane was confirmed after the membrane had been incubated with enzyme-conjugated secondary antibody, but before the enzyme–substrate reaction. Treatment of the nitrocellulose membrane dots with TCA produced the characteristic yellow-orange color for DMT. Moreover, material eluted from the TCA-treated dots with 50% acetonitrile exhibited the 495-nm absorbance peak characteristic of DMT (data not shown). TCA removal of DMT from the standard oligomers, before cross-linking of the oligomers to the membrane, resulted in negative dot-blot assays (data not shown). To further assess the specificity of the DMT MAb, the polysaccharide cyclodextrin

TABLE 1

Specificity of Monoclonal Antibody Recognition of Protecting Groups in Standard and Negative Control Oligonucleotides Determined by Dot Blot Assay

	Dot density (INT mm²)			
Oligomer[a]	Bz-MAb	Ibu-MAb	Ipr-Pac-MAb	DMT-MAb
Oligo Bz-dC	**0.63**	0.06	0.03	0.03
Oligo Bz-dA	**0.54**	0.05	0.04	0.04
Oligo dC^{Bz}	0.05	0.05	*[b]	0.02
Oligo dA^{Bz}	0.03	*	*	0.03
Oligo Ibu-dG	0.05	**0.53**	**0.57**	0.04
Oligo Ibu-dA	0.05	**0.49**	**0.58**	0.03
Oligo Ibu-dC	0.04	**0.45**	**0.57**	0.02
Oligo dG^{Ibu}	0.03	0.05	0.05	0.02
Oligo dA^{Ibu}	*	0.05	0.04	*
Oligo dC^{Ibu}	*	0.04	0.03	*
Oligo Ipr-Pac-dG[c]	0.02	0.04	**0.61**	0.02
Oligo $dG^{Ipr-Pac}$	0.04	0.03	0.04	0.03
Oligo DMT-dT	0.05	0.06	0.04	**0.68**
Oligo Trebler-dT	0.04	0.04	0.04	**0.83**
Oligo dT	0.02	0.03	0.03	0.03
DMT-chloride[c]	*	*	*	0.70

[a] Oligomers were cross-linked to the nitrocellulose membrane in equal amounts (32 pmol/dot). The amount of Ipr-Pac was estimated from protected nucleoside's extinction coefficient.

[b] Not determined.

[c] A solution of DMT-chloride was spotted to nitrocellulose at 0.25 A_{495}/dot, 32 pmol.

TABLE 2

Comparison of Oligonucleotide Protecting Group Detection by the MAb-Based Dot-Blot Assay, HPLC Nucleoside Composition, and UV/Visual Spectroscopy

	HPLC/spectroscopy[a]			MAb dot blot
Reference oligomer	Protected nucleotide (%)	Protecting group/ A_{260} of polymer (nmol)	Lowest detection limit of protecting group (nmol)	Lowest detection limit of protecting group (pmol)
Oligo Bz-dC	97.0	58.3	2–5	16–32
Oligo Bz-dA	88.0	76.8	2–5	16–32
Oligo Ibu-dG	92.0	21.1	2–5	8–16
Oligo Ipr-Pac-dC	ND[b]	ND	2–5	32[c]
DMT-dT	*[d]	11.3	0.9	11
Trebler-dT[e]	*[d]	22.8	2.3	28

[a] Amounts of Bz, Ibu, and Ipr-Pac were determined with HPLC nucleoside composition and UV analysis of enzyme-hydrolyzed DNA (0.5–1.5 nmol); amounts of DMT and Trebler were determined by visible spectroscopy (498 nm) with a DMT-chloride standard and confirmed by MALDI-MS.

[b] ND, not determined due to lability of Ipr-Pac under conditions of the HPLC nucleoside composition analysis.

[c] Estimated amount of Ipr-Pac assuming 1 A_{260} = 25 μg of polymer and the sample is 100% protected.

[d] Not determined.

[e] Trebler is the designation for a 5'-protection agent with three DMT moieties.

(Sigma Chemical Co.) was prepared in our laboratory to have multiple DMT groups and then bound to nitrocellulose membranes for a dot-blot assay. The DMT MAb recognized the DMT-derivatized cyclodextrin, whereas cyclodextrin itself gave rise to negative responses (data not shown).

Sensitivity and Quantification of the MAb Dot Blot of Protecting Groups

The MAb dot-blot assay provided a highly sensitive assessment of the amount of each protecting group (Fig. 2 and Table 2). However, commercial oligonucleotide products may contain trace amounts of protected nucleosides due to an incomplete deprotection after oligomer synthesis. Using the MAb dot-blot assay, detection of trace amounts of the Bz, Ibu, and Ipr-Pac protecting groups in DNA samples was compared to and validated by the UV-monitored, HPLC nucleoside composition analysis. In addition, the MAb dot-blot assay detection of the 5'-terminal DMT moiety was compared to and validated by visual spectroscopy. Standard curves of DMT versus absorbance were generated from visual spectroscopy of DMT-chloride. The lower limit of HPLC detection was 2–5 nmol of Bz-, Ibu-, or Ipr-Pac-protected nucleoside and 1–2 nmol of DMT or Trebler (Table 2). In these experiments, protected nucleosides were detected in the presence of 10 times as many deprotected nucleosides (Table 2). If the protected nucleoside in the hydrolyzed sample was below the 2–5 nmol detection limit of the HPLC nucleoside composition analysis, as much as 10 times the amount of hydrolyzed DNA could be applied to the column. However, the lower limit of detection would still be 2–5 nmol and more importantly, the HPLC column would have to retain the capacity and resolution required for the analytical separation of increasing amounts of dA, dC, and dG from their protected counterparts. In contrast, the lower limit of MAb detection using the same DNA samples was 8–32 pmol of protected nucleoside in the presence of 10,000-fold excess of deprotected nucleoside (Table 2). Thus, the MAb dot blot assay was able to detect protecting groups at the lower limit of 0.01% for these particular samples.

To further evaluate the ability of the MAb to detect trace amounts of protected nucleoside within vast amounts of deprotected nucleosides, samples of oligo Bz-dC were extensively diluted with the negative control oligo dC^{Bz}. Then, the samples were analyzed for the protected nucleoside using the dot-blot assay. The Bz-MAb was able to direct 8–16 pmol of Bz-protected nucleoside in the presence of 320 nmol of deprotected nucleoside (16 nmol of the 20-mer dC^{Bz}; Fig. 3). Furthermore, when Bz-dC oligomers containing 16, 32, and 64 pmol of the Bz-protected nucleoside were diluted with oligo dC^{Bz} containing 320, 640, and 1280 nmol of the deprotected nucleoside, respectively, the Bz-MAb was capable of detecting the Bz groups in each of the samples (data not shown). Assessments of Ipr-Pac-MAb, Ibu-MAb, and DMT-MAb demonstrated comparable sensitivities in detection of their respective protecting groups (data not shown). Thus, the MAb assay is able to detect picomole quantities of the individual protecting groups on nucleic acid samples even in presence of 20,000 times as many deprotected nucleosides.

Identification and quantification of the protecting groups by the MAb dot-blot assay was validated by HPLC nucleoside composition analysis. The oligos

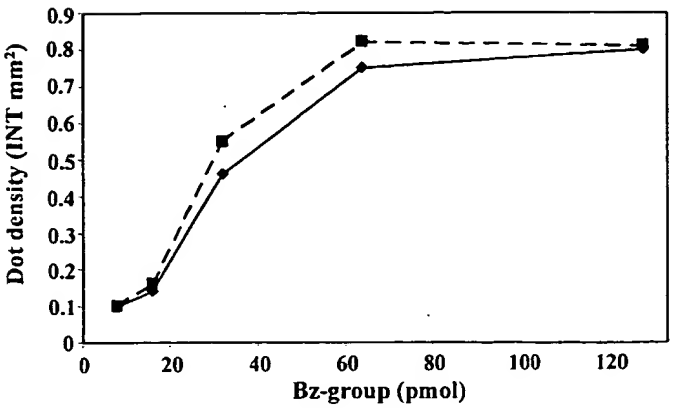

FIG. 3. Relationship between amount of protecting group and dot density of the MAb dot-blot assay. Oligo Bz-dC was applied to the nitrocellulose membrane according to the amount of Bz groups present, 8–124 pmol Bz per dot, as determined by the HPLC nucleoside composition analysis. The squares indicate the densities achieved with the Bz-MAb recognition of these samples. The diamonds indicate the densities achieved with the same samples but each diluted with a fixed amount (16 nmol DNA/dot) of negative control oligo dC^{Bz}.

Bz-dC and Bz-dA with 5 nmol of Bz group each, as determined by HPLC composition analysis, were mixed with the control oligo dC^{Bz} and oligo dA^{Bz}, respectively, to produce samples containing 5% protected oligomers. The samples were serially diluted and the amount of protection was determined by the nitrocellulose membrane dot-blot assay and by HPLC nucleoside composition analysis. After incubation with the Bz-MAb, dot-blot intensities were determined and the Bz-group amount was calculated by comparison with the corresponding oligo Bz-dC and oligo Bz-dA relative to corresponding negative oligomers. The detected amounts of Bz groups by the MAb dot-blot assay were 5.98 ± 0.36 and 5.11 ± 0.25 nmol, respectively. In comparison, the HPLC nucleoside composition analysis performed on the same samples detected 4.21 ± 0.01 and 10.92 ± 0.37 nmol of Bz groups. Thus, the MAb quantification of nanomole amounts of remaining protecting groups was within a factor of 2 of that determined by HPLC.

Identification and Quantification of Protecting Groups Remaining in Commercial Oligonucleotide Products

Two oligonucleotide products, deprotected and desalted, were purchased from each of eight different nucleic acid synthesis companies. To test whether these products contained protected nucleosides, equal amounts (absorbance at 260 nm) of the samples were UV cross-linked to nitrocellulose membranes and the membranes were incubated with the Bz-MAb, Ipr-MAb, and Ibu-MAb. Among the 16 20-mer samples, 2 with 10 repeated oligo dA–dC sequences were positively recognized with the Bz-MAb and, thus, identified as having been incompletely deprotected (Fig. 4). According to a comparison with the oligo Bz-dC and Bz-dA standards, one product contained 264 pmol of Bz group per A_{260} unit or about 5.2% contamination, and the other contained 54 pmol per A_{260} unit or about 1.0% contamination. After alkali treatment of the two samples, the dot-blot analysis was negative (data not shown). Oligomer products with the same sequence purchased from the same two companies at a later date yielded similarly positive results (data not shown). An oligo dA–dC sample from a third company exhibited a weak positive response with the Bz-MAb (Fig. 4). Neither the Ipr-Pac-MAb nor the Ibu-MAb reacted with the oligo dA–dC samples (Fig. 4). Two samples with 10 repeats of the dG–dT sequence were recognized by the Ipr-Pac-MAb and, thus, deemed incompletely deprotected of either Ipr-Pac or Ibu (Fig. 5). Because the Ibu-MAb did not recognize any of the dG–dT samples (Fig. 5), the Ipr-MAb must have reacted with incompletely deprotected Ipr-Pac groups. The Bz-MAb did not recognize the dG–dT samples (Fig. 5).

DISCUSSION

Monoclonal antibody identification and quantification of the protecting groups remaining on synthesized oligomers has several demonstrated advantages over conventional detection methods. The MAb dot-blot assay and ELISA were able to identify specific protecting groups remaining on oligonucleotides. Each MAb identified a specific protecting group remaining on the intact oligonucleotide independent of the base or sugar. Though CE and MS analyses of chemically synthesized

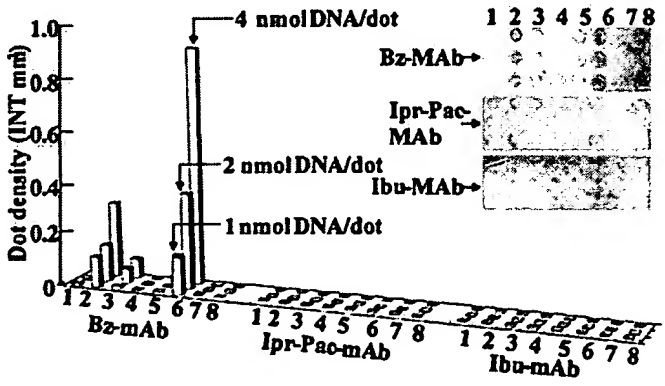

FIG. 4. Detection of protecting groups remaining in eight commercial samples of oligo dA–dC. A dot-blot assay was used to detect incompletely deprotected oligonucleosides obtained from eight different commercial sources (numbered 1–8). The oligonucleosides were composed of 10 repeats of dA–dC. The inset in the upper right corner presents the dot-blot results. In the bar graph, dot densities are shown for each sample in three different amounts (1, 2, or 4 nmol DNA per dot) probed with each of three antibodies, Bz-MAb, Ipr-Pac-MAb, and Ibu-MAb.

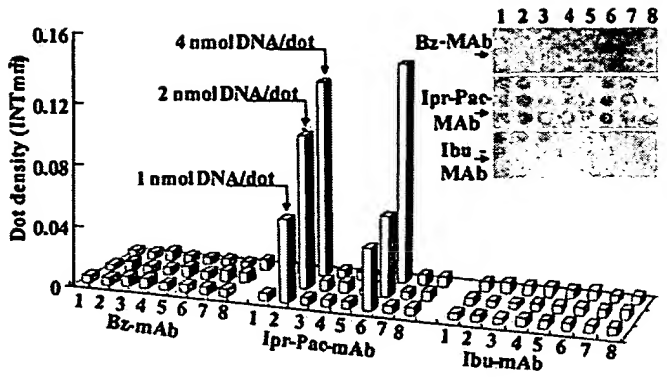

FIG. 5. Detection of protecting groups remaining in eight commercial samples of oligo dG–dT. A dot-blot assay was used to detect incompletely deprotected oligonucleosides obtained from eight different commercial sources (numbered 1–8). The oligonucleosides were composed of 10 repeats of dG–dT. The inset in the upper right corner presents the dot-blot results. In the bar graph, dot densities are shown for each sample in three different amounts (1, 2, or 4 nmol DNA per dot) probed with each of three antibodies, Bz-MAb, Ipr-Pac-MAb, and Ibu-MAb.

oligonucleotides distinguish full-length from aborted sequences (14–16), present methods do not detect and identify remaining protecting groups. In contrast, HPLC nucleoside composition analysis (13) is capable of identifying protected nucleosides in DNA or RNA samples. The HPLC analysis distinguishes specifically protected nucleosides by retention time and UV spectrum and, thus, requires enzyme hydrolysis of a portion of the sample to the constituent mononucleosides (13). However, some protecting groups will inhibit the enzymatic digestion, particularly the 2′-OH protection in RNAs, resulting in dinucleoside monophosphates that are difficult to identify. Whereas the MAb dot-blot assay and ELISA analyze 96 samples per membrane or microplate, the HPLC sample injection though automated requires approximately an hour per sample. Thus, MAbs are capable of identifying protecting groups remaining on multiple DNA and RNA oligonucleotide samples using standard immunological assays.

The MAb dot-blot assay and ELISA were able to detect a single protected nucleoside in DNA and RNA samples containing 20,000-fold deprotected nucleosides. Crowding either by adjacent protecting groups or by the binding of one MAb molecule interfering with the binding of an adjacent, second antibody could affect quantification. However, protecting-group detection and quantification on the homopolymer DNA standards were verified by HPLC, indicating little to no steric hindrance. Possible negative cooperativity in binding due to adjacent protecting groups in heteropolymeric oligomers or by already present MAb needs to be determined. Position of protecting groups either at the middle or at the termini of the oligonucleotide and sequence context also may affect the quantification.

The MAb assessment of protecting groups detected and quantified 8–32 pmol of protected nucleoside in the presence of thousands of nanomoles of deprotected nucleosides without hydrolysis or other treatment of the sample. With the use of purified, fluorescein-conjugated antibody preparations and highly optimized experimental conditions, the dot-blot assay should become even more sensitive. In comparison, UV-monitored HPLC assessment of DNA hydrolysates was limited to the identification and quantification of nanomole quantities of protected nucleoside within sample sizes that would not affect column resolution and peak shape, approximately 100–200 nmol of DNA or RNA mononucleosides.

Additional advantages to a MAb detection method include reduced instrument and labor costs and the ability to adapt the assays (dot blot or ELISA) to robotics capable of processing hundreds to thousands of samples in several hours. If protecting groups are detected in a nucleic acid sample, the sample can be re-treated for deprotection and retested for remaining protecting groups, as we have demonstrated. This precludes the need to dispose of expensive samples. Presumably, the MAb reagents provide the advantage that nucleic acids synthesized on, or attached to, solid supports such as DNA arrays on chips could be tested for remaining protecting groups while on the solid support. An expensive array with remaining protecting groups need not be discarded; one needs only to be cognizant of the sequences that retain protecting groups.

Although not demonstrated here, another advantage of the MAbs is their potential use in purifying oligonucleotides. By employing antibody-affinity chromatography methods and MAbs against the base- and 2′-OH-protecting groups, affinity chromatography could be used to separate incompletely deprotected nucleic acids from completely deprotected molecules and full-length product from aborted sequences.

Another advantage to the use of MAb reagents in the identification and quantification of remaining protecting groups, and to their use for purification of oligomers, is their group specificity. Because the MAb reagents are group specific and not influenced by the polymer support as demonstrated for the identification of DMT groups on cyclodextrin, the technology is applicable to protecting groups used in the synthesis of other polymers. The chemical synthesis could be sequential, such as the synthesis of nucleic acids and peptides, or nonsequential, including that of dentrimers. As long as protecting groups are used in the polymer synthesis to influence the direction of the coupling and/or to inhibit reactivity of particular functional groups, the MAbs could be used for detection of pro-

tecting groups in the product and in purification of the polymer.

Chemically synthesized oligonucleotides have been used extensively in molecular biology research and drug discovery. With chemically synthesized DNA or RNA oligomers in 10-fold excess, any incompletely deprotected nucleic acid that constitutes 10% or less is of little concern for assays *in vitro*. However, notable exceptions perhaps are the X-ray crystallography and NMR determinations of structure and structural interactions. Only recently, oligonucleotides have been synthesized and tested as therapeutics through their abilities to control specific gene expression as antisense molecules and as diagnostics through their ability to identify the presence and expression of specific genes, via DNA array technologies. In contrast to their use in molecular biology, quality control of oligonucleotide therapeutics and diagnostics is critical to their use as antisense drugs and as tools in the array analysis of gene expression. MAb detection, identification, and quantification of oligonucleotide-protecting groups could provide the specificity and sensitivity and application to hundreds of individual samples required of antisense therapeutics and array technology diagnostics. The MAb dot-blot assay and ELISA are suitable for university and company oligonucleotide synthesis facilities, as well as the pharmaceutical industry's quality control of nucleic acid therapeutics and diagnostics.

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EXHIBIT C

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QC in antisense oligo synthesis

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To the editor:

Interest in oligonucleotide antisense therapeutics has regained momentum[1, 2]. One antisense therapeutic, Vitravene, has been approved, 12 are in clinical trials[1], and others are in various planning stages[3]. High-quality chemical synthesis of antisense oligonucleotides via nucleobase and sugar-protected phosphoramidites is crucial to the expectations of low toxicity, reduced side effects, and low costs[2]. However, neither the coupling reaction producing the growing polymer chain nor the subsequent deprotection of the full-length oligonucleotide occurs with 100% efficiency[4]. Thus, quality and regulatory concerns about antisense therapeutics have been expressed by scientists at the Food and Drug Administration (FDA;

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Rockville, MD)[5].

Incomplete deprotection of nucleoside-reactive groups could be responsible for the unexplained results observed in the early *in vitro* and cellular stages of drug discovery[2]. It could also be responsible for immunological responses seen at high doses in animal models and clinical trials[2] and thus contribute to erroneous conclusions about drug efficacy. The protecting groups, and not the nucleic acid itself, can elicit an antibody response[6].

Unfortunately, no simple, reproducible, sensitive, and inexpensive analytical method exists to identify and quantify every protecting group that may remain in an antisense sample. High-performance liquid chromatography (HPLC) nucleoside composition analysis identifies and quantifies protecting groups remaining on oligonucleotides. However, the analysis is insensitive because it depends on enzymatic cleavage of the oligonucleotide and on UV diode-array detection for identification and quantification. Capillary electrophoresis and mass spectrometry detect the aborted sequences, but are not easily adapted to identifying and quantifying the protecting groups that remain on the oligonucleotide[6].

To address this problem, we previously developed monoclonal antibodies (mAbs) for the specific identification and quantification of the nucleobase and sugar protecting groups commonly used in DNA and RNA chemical syntheses[6]. Using these mAbs, we now present a dot-blot assay and a microplate enzyme-linked immunosorbent assay (ELISA) for identification and quantification of protecting groups that remain in standard, intact DNA and RNA oligonucleotide samples (Fig. 1).

The mAbs detect as little as 8 pmol of the specifically protected nucleoside in intact DNA or RNA composed of 160 nmol of the deprotected nucleoside. Thus, the mAb analysis is able to detect a single protected nucleoside in oligonucleotide samples containing 2×10^4 deprotected nucleosides. In contrast, HPLC nucleoside-composition analysis of enzyme-hydrolyzed DNA is limited to the detection of 2–5 nmol of protected nucleoside[6]. Using our present mAb dot-blot assay, 5 of 16 commercial DNA products obtained from eight different companies are found to have 1.0–5.2% contamination

from benzoyl- and isopropylphenoxyacetyl-protecting groups (Fig. 2A).

Monoclonal antibodies have the advantage of identifying specific protecting groups that remain on intact oligonucleotides independent of the base or sugar. The assays are amenable to robotic analysis of hundreds of samples and should be applicable to oligonucleotides on solid supports. The antibodies could be used for the synthesis of affinity columns to separate incompletely deprotected nucleic acid from completely deprotected molecules.

Because the mAb reagents are group specific and not influenced by the polymer support, as demonstrated by the identification of 4,4'-dimethoxytrityl groups on cyclodextrin (Fig. 2B), the technology can be applied to the detection of protecting groups remaining from the synthesis of other biopolymers, dendrimers, and biopolymers on solid supports, such as oligonucleotide and peptide arrays.

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